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# Designer small molecules to target calcium signalling

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## Abstract

Synthetic compounds open up new avenues to interrogate and manipulate intracellular  $\text{Ca}^{2+}$  signalling pathways. These may ultimately lead to drug-like analogues to intervene in disease. Recent advances in chemical biology tools available to probe  $\text{Ca}^{2+}$  signalling are described, with a particular focus on those synthetic analogues from our group that have enhanced biological understanding or represent a step towards more drug-like molecules. Adenophostin (AdA) is the most potent known agonist at the inositol 1,4,5-trisphosphate receptor ( $\text{IP}_3\text{R}$ ) and synthetic analogues provide a binding model for receptor activation and channel opening. 2-*O*-Modified inositol 1,4,5-trisphosphate derivatives that are partial agonists at the  $\text{IP}_3\text{R}$  reveal key conformational changes of the receptor upon ligand binding. Biphenyl polyphosphates illustrate that simple non-inositol surrogates can be engineered to give prototype  $\text{IP}_3\text{R}$  agonists or antagonists and act as templates for protein co-crystallization. Cyclic adenosine 5'-diphosphoribose (cADPR) can be selectively modified using total synthesis, generating chemically and biologically stable tools to investigate  $\text{Ca}^{2+}$  release via the ryanodine receptor (RyR) and to interfere with cADPR synthesis and degradation. The first neutral analogues with a synthetic pyrophosphate bioisostere surprisingly retain the ability to release  $\text{Ca}^{2+}$ , suggesting a new route to membrane-permeant tools. Adenosine 5'-diphosphoribose (ADPR) activates the  $\text{Ca}^{2+}$ -,  $\text{Na}^{+}$ - and  $\text{K}^{+}$ -permeable Transient Receptor Potential Melastatin 2 (TRPM2) cation channel. Synthetic ADPR analogues provide the first structure-activity relationship (SAR) for this emerging messenger, and the first functional antagonists. An analogue based on the nicotinic acid motif of nicotinic acid adenine dinucleotide phosphate (NAADP) antagonizes NAADP-mediated  $\text{Ca}^{2+}$  release *in vitro* and is effective *in vivo* against induced heart arrhythmia and autoimmune disease, illustrating the therapeutic potential of targeted small molecules.

## The molecules that stimulate $\text{Ca}^{2+}$ release

$\text{Ca}^{2+}$  Regulates many cellular events and plays a key role in processes as diverse as cell division, muscle contraction, cell death and fertilization [1]. The spatial and temporal release of  $\text{Ca}^{2+}$  within the cell is tightly controlled by second messengers. In the thirty years since *myo*-inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) was shown to release  $\text{Ca}^{2+}$  from internal stores [2], the importance and complexity of  $\text{IP}_3$ -regulated signalling pathways and the  $\text{IP}_3$  receptor ( $\text{IP}_3\text{R}$ ) have been revealed. More recently, three adenosine nucleotide-based second messengers cyclic adenosine 5'-diphosphoribose (cADPR), nicotinic acid adenine dinucleotide phosphate (NAADP) and adenosine 5'-diphosphoribose (ADPR) have been discovered [3]. In humans all three are formed from nicotinamide adenosine 5'-dinucleotide ( $\text{NAD}^{+}$ ) by the multifunctional ADPR-cyclase CD38 [4]. They mobilize  $\text{Ca}^{2+}$  using pathways unrelated to  $\text{IP}_3$ .

These four  $\text{Ca}^{2+}$ -releasing second messengers are all highly specific ligands for their intramolecular ion channel target. Each has highly polar phosphate groups that are negatively charged at normal physiological pH [5] and likely correspond to positively charged or electron poor residues in their binding counterpart, an interaction often presumed to be critical for their activity. It is therefore a major challenge for synthetic chemists to design molecules that not only retain the specificity of the natural messengers, but also overcome the inherent barriers for studying biological processes in whole cells and organisms. Still more complex is the challenge to achieve specificity in translating molecules based upon such ubiquitous messengers into models of disease.

## Small molecule design

Currently, there are very few synthetic examples of neutral, active, drug-like molecules able to modulate one of these critical pathways. The non-competitive NAADP antagonist Ned-19 was identified by virtual screening [6] and is a valuable tool for biological studies [7]. Recently the specific cardiac ADPR cyclase inhibitors SAN2589 and SAN4825 were identified by high-throughput screening [8].

Our design-based approach to chemical biology tools aims to address the following considerations:

1) *Membrane permeability to access intracellular receptors in whole cell systems*: Multiple negatively charged phosphate groups give the natural messengers poor membrane permeability. The synthesis of caged analogues of NAADP, cADPR and IP<sub>3</sub>, where the phosphates are masked as ester derivatives or with photolabile groups, has addressed this to some extent [9-11]. The advantage of this strategy is that the exact natural ligand is released by intracellular esterases or upon irradiation. However, frequently the rate of release of the active compound is slow, particularly when multiple phosphates are involved, and is in competition with degradation to unwanted side products. Additionally, the modified analogues are not always chemically homogenous species. More recently, bioisosteric approaches [12] have successfully generated active neutral compounds making these an attractive alternative.

2) *The complexity of the natural Ca<sup>2+</sup> signalling molecules*: This frequently results in arduous multi-step synthetic routes and limits the scope, yield and number of analogues that can be prepared. Replication of biologically simple processes can be very difficult in a synthetic chemistry setting. This makes the search for useful biological tools demanding of time and resources. Simplified structures with shorter synthetic routes are appealing as they may be more quickly prepared, evaluated and optimized.

3) *The transient nature of the second messengers*: This makes the natural compounds difficult to work with in biological studies as they may be unstable in aqueous solution or sensitive to physiological conditions (e.g. IP<sub>3</sub> is a substrate for kinases and phosphatases, cADPR is readily hydrolyzed by CD38). By introducing chemical and biological stability, we can provide reliable tools that may be studied in isolation without interference from degradative pathways.

4) *The large size and hydrophilic nature*: This is particularly true for the adenosine nucleotide messengers (e.g. NAADP has a molecular weight of 745). Generally, this does not translate well into a good drug and it exacerbates the difficulties of multistep, low yielding synthetic routes. Systematic studies to determine the structure-activity relationship (SAR) of the natural molecule can help to identify critical motifs and reduce the mass of a designed related ligand.

5) *Selectivity and specificity for the desired ion channel*: This must be in preference to a number of similar ion channels that interact with highly similar species. Ligand design based on biological information, such as crystal structures where available, ensures that the affinity of a ligand is tuned to maximize interactions with its specific ion channel target.

## Agonists and antagonists for the IP<sub>3</sub>R

Many analogues of IP<sub>3</sub> have been synthesized to study SAR at the IP<sub>3</sub>R [13, 14]. In 1993, the glyconucleotide adenophostin A (AdA) was isolated from a culture broth of *Penicillium brevicompactum* [15]. It is an IP<sub>3</sub>R agonist with at least ten times greater potency and affinity than IP<sub>3</sub>. Its potency, the fact that it is not degraded by the enzymes that metabolize IP<sub>3</sub>, and the possibility of introducing synthetic modifications makes preparation of AdA analogues attractive, both as carbohydrate [16] and nucleotide derivatives [17]. Understanding interactions at the IP<sub>3</sub>R may ultimately lead to the rational design of potent ligands.

A comparison of chemical structures suggests that the 3'',4''-bisphosphate of AdA may mimic the 4,5-bisphosphate of IP<sub>3</sub>, and that perhaps the AdA 2'-phosphate group is better positioned to interact with the

IP<sub>3</sub>R binding site than the IP<sub>3</sub> 1-phosphate (Fig. 1A). However, synthesis of IP<sub>3</sub> analogues in which the 1-phosphate is positioned further from the ring (e.g. by two carbons) did not increase activity [18]. Preparation of an orthogonally protected disaccharide intermediate permitted introduction of modified bases and aromatic groups at C1' to explore the effect of the adenine ring system on activity [19]. No analogue without an adenine base is more potent than IP<sub>3</sub>, suggesting that the adenine has a critical interaction with the IP<sub>3</sub>R. Modifications at N6 and C2 of the base generated additional SAR information about the shape and tolerance of the binding site (Fig. 1A).

The bisphosphate analogues of AdA were synthesized via three bespoke routes to analyze the role of each phosphate group in binding to the IP<sub>3</sub>-binding core (IBC) of the IP<sub>3</sub>R. The IBC consists of  $\alpha$ - and  $\beta$ -domains which operate like a clam, the closure of which activates channel opening [20]. The contrasting agonist activity of these analogues at the IP<sub>3</sub>R led to a proposed binding model in which any active analogue must engage both domains (Fig. 1B). Furthermore, the activity of 2'-dephospho-AdA (107 nM at IP<sub>3</sub>R1, *cf.* 23 nM for IP<sub>3</sub>) [20] suggests it is possible to design less polar IP<sub>3</sub>R agonists. At their simplest, future analogues might consist of a simple linker connecting groups to interact with each of the  $\alpha$ - and  $\beta$ -domains. In combination with mutational analysis, a proposed important cation- $\pi$  interaction between the adenine and Arg504 has been suggested to rationalize the enhanced potency of AdA (Fig. 1C). In support of this idea, mutation of this residue causes a much greater reduction in AdA affinity, compared to IP<sub>3</sub> [21, 22].

Synthesis of 2-*O*-modified IP<sub>3</sub> analogues (e.g. Fig. 1D) revealed key steps in IP<sub>3</sub>R activation. As partial agonists, they highlighted the role of a conformational change in the *N*-terminal domain to transmit information to the pore for channel opening [23]. Conjugation with fluorescein isothiocyanate (FITC) via an aminoethyl linker at the 2-position of IP<sub>3</sub> gave the fluorescent analogue, FITC-IP<sub>3</sub>. This high affinity partial agonist was used to probe the thermodynamics of ligand binding using fluorescence polarization, establishing that the IBC alone is sufficient for the higher affinity binding of AdA, compared to IP<sub>3</sub>, but that the two ligands have different entropy and enthalpy contributions for binding [24].

More recently, non-inositol based aromatic polyphosphates have provided a route to avoid some of the difficulties of IP<sub>3</sub> and AdA synthesis, and show the potential to mimic interactions at the IP<sub>3</sub>R and elsewhere [25]. These simple achiral molecules are attractive for future analogue design, and often also remove the problem of differentiation between multiple hydroxyl groups and associated phosphate migration during synthesis. The IP<sub>3</sub>R antagonist biphenyl 2,3',4,5',6-pentakisphosphate was generated in only two steps (Fig. 1E) [26]. Synthesis of biphenyl hexakisphosphate analogues illustrated that repositioning one phosphate group on the aromatic ring can induce an effective IP<sub>3</sub>R agonist/antagonist switch [27]. These compounds have also shown potential as templates for co-crystallization, facilitating the first high resolution structure of Src-homology 2 domain-containing inositol phosphatase 2 (SHIP2, Fig. 1F) [28]. Future iterations of such simple analogues will need to focus on reducing polarity to improve permeability and specificity over the related 5-phosphatases. However, the simplicity and versatility of the chemistry to access them implies that optimization should be straightforward.

### Probing Ca<sup>2+</sup> release at the ryanodine receptor (RyR) with cADPR analogues

cADPR Effects Ca<sup>2+</sup> release directly or indirectly via the RyR [29], and analogues may provide agonist or antagonist tools to help elucidate the mechanism by which this occurs [30]. Since cADPR is readily hydrolyzed in both aqueous solution and under physiological conditions, stable analogues are highly desirable [31]. Initially, a chemoenzymatic route was employed to cyclize the respective NAD<sup>+</sup> analogue using ADP-ribosyl cyclase from *Aplysia californica*. A 6-NH<sub>2</sub>  $\rightarrow$  6=O substitution on the base gives a more chemically and biologically stable analogue, cIDPR, with an equivalent Ca<sup>2+</sup>-releasing ability in T-lymphocytes (Fig. 2B) [32], and this scaffold has been widely used in analogue design (Fig. 2A).

To promote cyclization of the corresponding cIDPR precursor at N1, rather than N7, modification of the base at C8 is required [32]. This illustrates the limitations of using a chemoenzymatic route, but also led to the

discovery of the first membrane permeant cADPR agonist, 8-Br-cIDPR (Fig. 2C) [33]. Total synthesis routes have frequently required modification of the *N*1-ribose to improve analogue tractability and stability, for example in cADP-carbocyclic ribose [34], cIDPRE and cIDPDE [35, 36]. A recent review covers related analogues and their effect on  $\text{Ca}^{2+}$  activity in T-cells [37]. We have developed a total synthetic route to cIDPR analogues [38] that allows independent functionalization of each structural motif. Replacing the *N*9-ribose with a butyl chain improves the stability of the *N*9-link and should improve membrane permeability (Fig. 2A). These analogues inhibit hydrolysis of cADPR by CD38 and this may provide future opportunities for upstream  $\text{Ca}^{2+}$  modulation [39]. Furthermore, crystallization of 8- $\text{NH}_2$ -*N*9-butyl-cIDPR with CD38 catalytic domain revealed a potential mechanism for cADPR hydrolysis by the cyclase (Fig. 2D). Replacing the negatively charged pyrophosphate group is attractive in the quest for more drug-like, membrane permeant tools. The use of 'click-chemistry' generated the first neutral analogue of cADPR in which the phosphate is replaced with an uncharged bioisosteric 1,4-triazole (Fig. 2A) [40]. The surprising, albeit weak, activity of these first analogues in  $\text{Ca}^{2+}$ -release (Fig. 2E) suggests that the cADPR receptor is a potentially druggable target. To assess the minimum structural requirement for activity, smaller fragment analogues (*N*1-inosine monophosphates, *N*1-IMP, Fig. 2A) were designed, based on the structures of CD38 co-crystallized with cIDPR analogues [41]. *N*1-IMP were accessed via acidic hydrolysis of the parent cyclic compound and are low  $\mu\text{M}$  inhibitors of cADPR hydrolysis by CD38. They represent a promising lead to simpler low molecular weight inhibitors.

### Activation of the TRPM2 cation channel by ADPR

ADPR activates Transient Receptor Potential Melastatin 2 (TRPM2), a  $\text{Ca}^{2+}$ -permeable cation channel, and has only recently been considered a second messenger [42]. An important role for TRPM2 has been suggested in cells of the innate immune system [43]. The first SAR study of ADPR combined synthetic chemistry and chemoenzymatic methods to access analogues that were selectively modified in each of the four main structural motifs (Fig. 3A) [44].

Analogues were evaluated by whole cell patch clamp experiments using HEK293 cells transfected with TRPM2 (Fig. 3B). Introduction of a hydrophobic substituent at the adenine 8-position caused a switch in activity to generate the antagonist 8-phenyl-ADPR (Fig. 3C) and further improvement was achieved in combination with a 2'-deoxy modification on the adenosine ribose to generate the most potent TRPM2 antagonist to date, 8-phenyl-2'-deoxy-ADPR ( $\text{IC}_{50} = 3 \mu\text{M}$ ). The former can be synthesized in only four steps from  $\text{NAD}^+$  by introduction of the 8-phenyl substituent to 8-Br-ADPR using Suzuki chemistry. 8-Phenyl-ADPR was shown to inhibit neutrophil migration, a downstream effect of TRPM2 activation (Fig. 3D) [44]. Weak antagonist activity was also seen when the pyrophosphate was substituted for a decorated sulfamate group, and this is a promising lead to more stable, less polar analogues of ADPR. However, useful analogues are still very much in their infancy for this application.

### Antagonists of the NAADP receptor

NAADP is the most potent endogenous  $\text{Ca}^{2+}$ -mobilizing compound known to date [45]. We synthesized NAADP via nicotinamide adenine dinucleotide phosphate (NADP) using a combined total synthesis and chemoenzymatic route [46]. NADP is inactive despite being structurally very similar to NAADP (Fig. 4A) [47]. Thus, the nicotinamide  $\rightarrow$  nicotinic acid switch appeared to offer an opportunity for chemical biological intervention in NAADP-mediated  $\text{Ca}^{2+}$  signalling. Indeed, co-injection of nicotinic acid, but not nicotinamide, greatly reduced  $\text{Ca}^{2+}$  signalling induced by NAADP [48]. Furthermore, whereas NAADP is large in size and hydrophilic, nicotinic acid is a more attractive starting point for development of a membrane permeant small molecule. Cellular uptake of nicotinic acid itself is slow, so alkylation with 2-bromo-acetamides was used as a starting point to generate a library of alkylated derivatives with more drug-like properties. BZ194 (Fig. 4B) emerged as an antagonist of  $\text{Ca}^{2+}$  signalling in Jurkat T cells, blocking both the  $\text{Ca}^{2+}$  peak and plateau when co-injected with NAADP (Fig. 4C). Importantly, BZ194 co-injected with  $\text{IP}_3$  or cADPR did not inhibit  $\text{Ca}^{2+}$

release, suggesting specificity for the NAADP pathway (Fig. 4D) [48]. Synthesis of BZ194 and related compounds is a high yielding two-step process that permits rapid preparation and evaluation of analogues, and this seems promising for future optimization.

The utility of BZ194 as an NAADP antagonist was recently illustrated *in vivo* in a model of multiple sclerosis [49]. Interference with the NAADP signalling pathway suppresses the formation of autoimmune inflammatory lesions and thus might qualify as a novel strategy for the treatment of T-cell mediated autoimmune diseases. Moreover, the compound was also effective in cardiac myocytes *in vitro* and isoproterenol-induced arrhythmias *in vivo* were ameliorated by prior injection of BZ194 (Fig. 4E) [50].

## Conclusion

Unravelling the mechanism by which a second messenger modulates its biological target is critical to understanding and ultimately intervening in  $\text{Ca}^{2+}$  signalling-related disease states. Although significant barriers must be overcome, synthetic chemistry can access functional molecules that modulate intracellular  $\text{Ca}^{2+}$  signalling both as mechanistic probes and prototype drug candidates. Strategies that combine rational design with chemical synthesis are best placed to deliver these powerful tools.

Chemical approaches have tended toward one of two extremes. Complex analogues that are structurally very similar to the natural ligand may be synthetically challenging, requiring multi-step routes that may only afford small amounts of valuable material for biological evaluation. However, the close similarity of such analogues to their natural counterparts can lead to biologically relevant binding models while co-crystallization with protein targets may provide invaluable structural and mechanistic insights. Another attraction of this approach, particularly for the mainstream organic chemist, is the opportunity to develop new synthetic chemistry during the design process. This can be expensive both in terms of time and quantity, meaning that subsequent scale-up to amounts needed for e.g. *in vivo* work is often difficult. However, in fortuitous instances work may lead both to independent novel chemistry *and* novel biology.

On the other hand, an approach using short routes to simple, active compounds can also be very attractive. Such routes may lack chemical elegance *per se*, but permit rapid generation of analogues for evaluation. Retention of specificity is critical to the utility of this strategy. The biphenyl polyphosphates, for example, can be easily synthesized and retain surprisingly diverse activities at their target receptor or enzyme; although in these prototypes, membrane permeability and selectivity is compromised. While initially synthetically simple, the real challenge here lies in the medicinal chemistry design and optimization processes to tailor-make the desired biological effects.

Drug-like properties are crucial for translation into early disease models. The first neutral cADPR analogues required a non-trivial synthesis but are new leads for simplified, membrane permeant modulators of  $\text{Ca}^{2+}$  release at the RyR. They suggest that activity can be retained when a phosphate or pyrophosphate is replaced with a bioisostere. By contrast, rational design of simple small analogues of nicotinic acid has afforded specific antagonists for the NAADP receptor that are stable, membrane permeant and amenable to very straightforward chemistry, with the potential for later scale-up. BZ194 thus fulfils many considerations for an ideal design-based probe and its activity in disease models *in vivo* illustrates the promise of such an approach in generating drug-like analogues for second messenger signalling pathways.

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## Key words

Calcium signalling, IP<sub>3</sub>, adenophostin A, cADPR, NAADP, chemical biology

## Abbreviations

AdA, Adenophostin A; ADPR, adenosine 5'-diphosphate ribose; cADPR, cyclic adenosine 5'-diphosphoribose; cIDPR, cyclic inosine 5'-diphosphoribose; FITC, fluorescein isothiocyanate; IBC, fMLP, formyl-methionyl-leucyl-phenylalanine; IBC, IP<sub>3</sub>-binding core; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; IP<sub>3</sub>R, IP<sub>3</sub> receptor; iso, isoproterenol; NAADP, nicotinic acid adenine dinucleotide phosphate; NAD<sup>+</sup>, nicotinamide adenosine 5'-dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; N1-IMP, N1-inosine monophosphate; RyR, ryanodine receptor; SAR, structure-activity relationship; SHIP2, Src-homology 2 domain-containing inositol phosphatase 2; TRPM2, Transient Receptor Potential Melastatin 2.

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## Figures

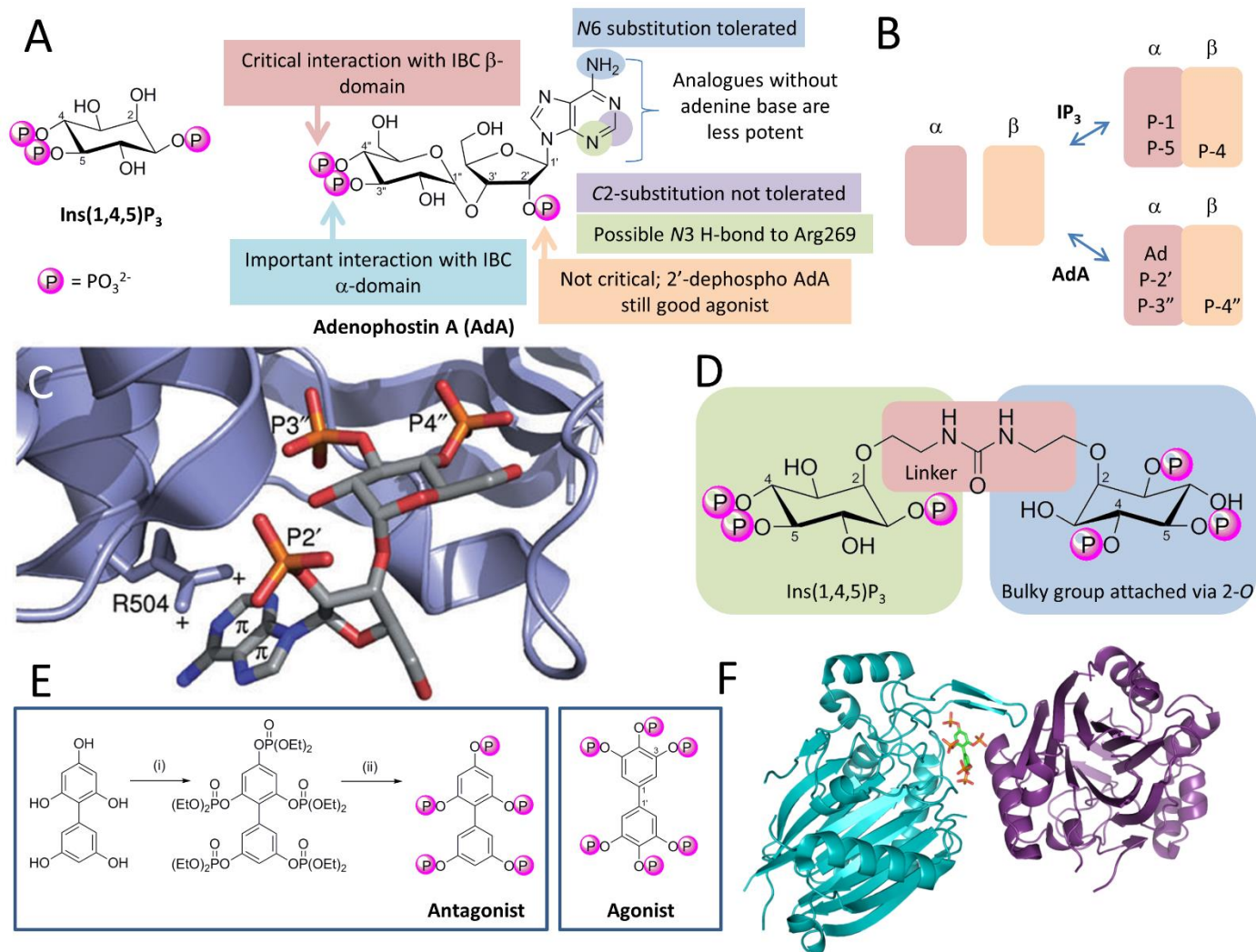


Figure 1: Agonists and antagonists of the IP<sub>3</sub>R

(A) The structure of IP<sub>3</sub> and AdA, SAR observations from synthetic AdA analogues; (B) Development of a rationale for the binding of the two ligands to the IP<sub>3</sub>R activating channel opening; requirement for both the  $\alpha$ - and  $\beta$ -domains to be engaged; adapted from [21] (C) Proposed model in which adenine has a cation- $\pi$  interaction with Arg504 in the IP<sub>3</sub>R binding site, adapted from [19]; (D) 2-O-modified ligands such as 2-O-linked dimeric IP<sub>3</sub> are partial agonists of the IP<sub>3</sub>R; (E) Simple benzene polyphosphates can be both antagonists and agonists of the IP<sub>3</sub>R, adapted from [27]; (F) Co-crystal structure of SHIP2 generated with lipid headgroup surrogate 2,3',4,5',6-pentakisphosphate (PDB code 4A9C) [28].

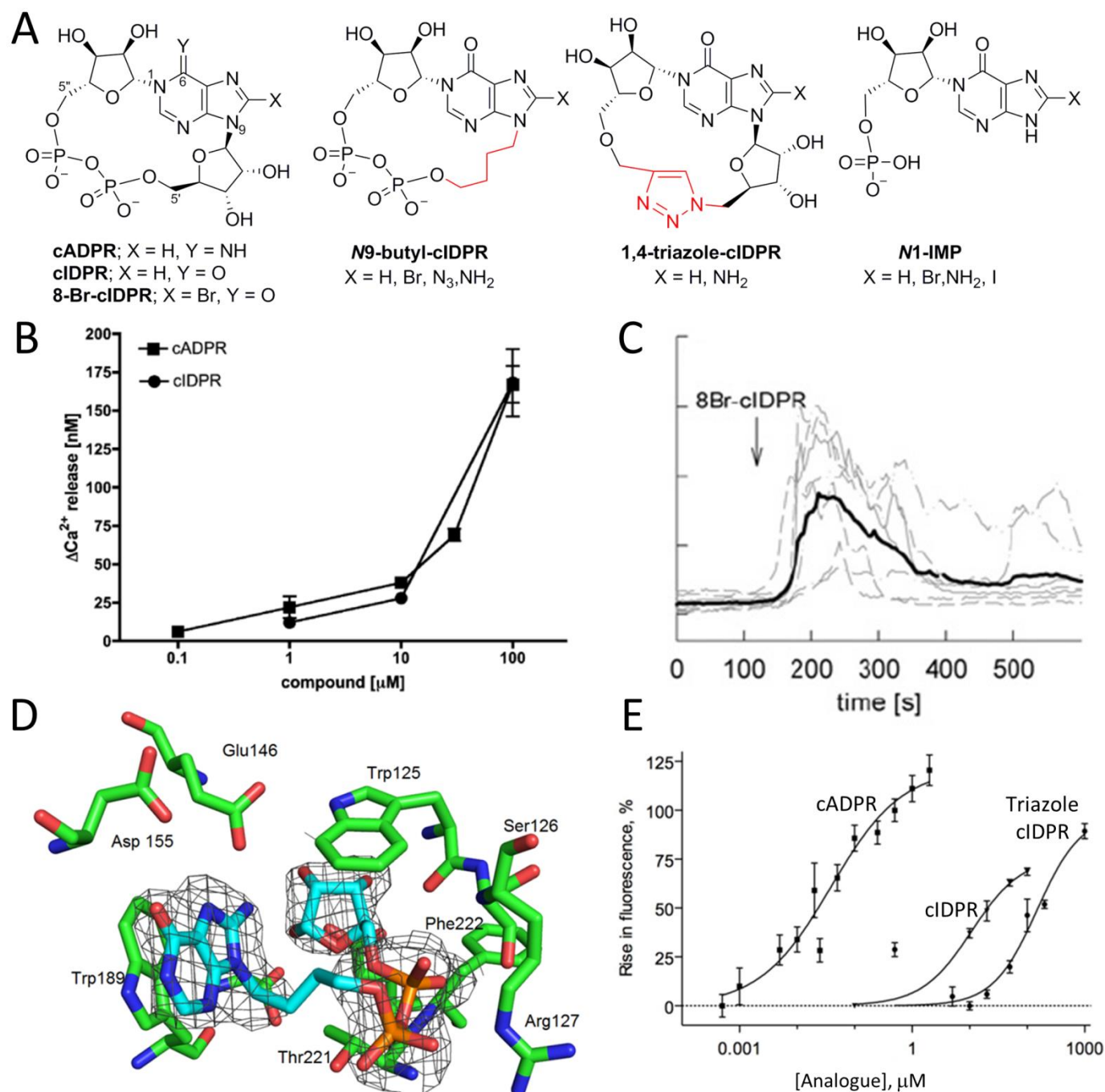


Figure 2: Analogues of cADPR

(A) Structure of cADPR, cIDPR and 8-Br-cIDPR, and new synthetic analogues; (B) cIDPR releases Ca<sup>2+</sup> with equivalent potency to cADPR in permeabilized T-cells, adapted from [32]; (C) 8-Br-cIDPR is a membrane permeant agonist in whole T-cells, adapted from [33]; (D) 8-NH<sub>2</sub>-N9-butyl-cIDPR co-crystallized with CD38 (PDB code 4TMF) [39]; (E) A neutral triazole-based cADPR analogue releases Ca<sup>2+</sup> in sea urchin egg homogenate, adapted from [40].

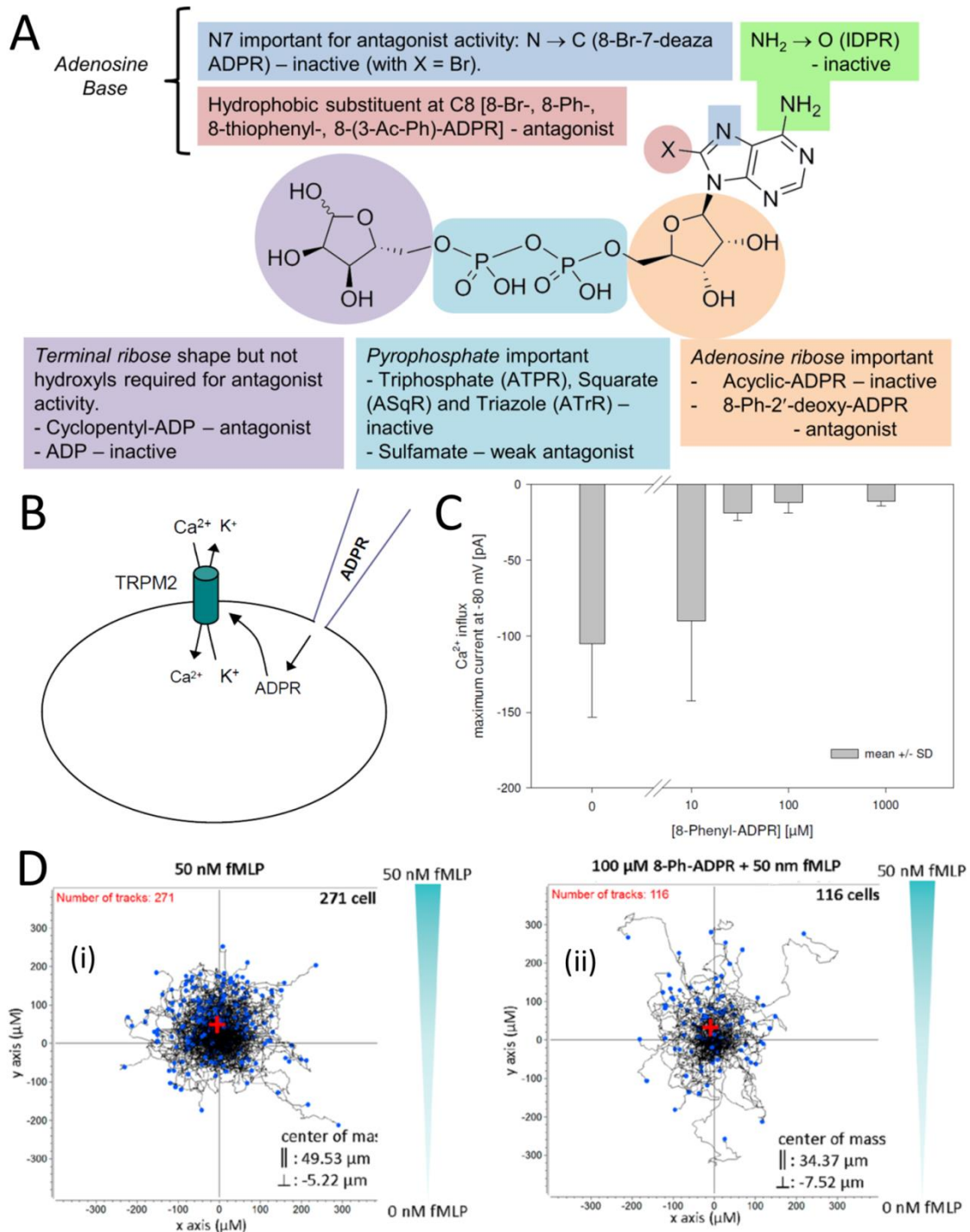


Figure 3: Exploring the SAR of ADPR at the TRPM2 cation channel

(A) Synthetic analogues generate the first SAR for ADPR at the TRPM2 channel, adapted from [44]; (B) ADPR analogues were studied by whole cell patch-clamp experiments to measure Ca<sup>2+</sup> and K<sup>+</sup> ion flow; (C) 8-Phenyl-ADPR is a potent antagonist (IC<sub>50</sub> = 11 μM) of ADPR (100 μM) induced Ca<sup>2+</sup> influx at TRPM2; (D) Mobility of neutrophils, previously shown to be a downstream effect of TRPM2 activation, is (ii) reduced by TRPM2 antagonist 8-phenyl-ADPR compared to (i) control; adapted from [44].

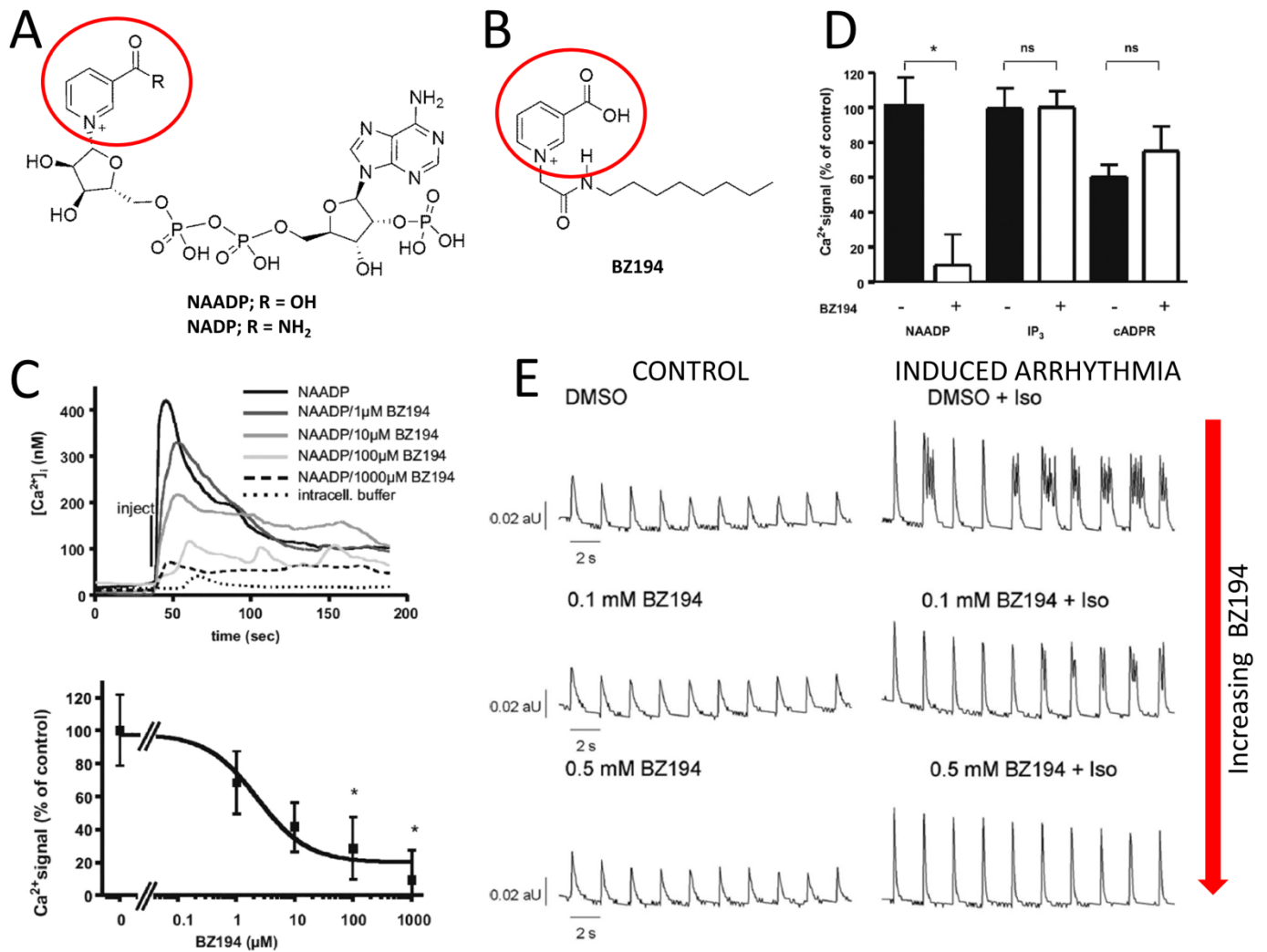


Figure 4: Design and activity of NAADP-receptor antagonist BZ194

(A) The structure of NAADP and NADP highlighting nicotinic acid/nicotinamide switch; B) Small molecule NAADP antagonist BZ194; c) Effect of BZ194 on NAADP-induced Ca<sup>2+</sup> signalling, adapted from [48]; D) BZ194 is a selective antagonist for NAADP-mediated Ca<sup>2+</sup> release [48]; E) Pre-injection of BZ194 ameliorates induced arrhythmia *in vivo*, adapted from [50]. Iso =  $\beta$ -adrenergic agonist isoproterenol.